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Copulation induces Arc expression in sex-relevant brain regions.

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Copulation induces Arc expression in sex-relevant brain regions.

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Dedication

To Jayson Bernal – I have no idea how I would have made it this far without all of your support and love.

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Abstract

Copulation induces Arc expression in sex-relevant brain regions.

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The study of copulation has contributed to knowledge of hormonal effects on behavior and natural reward mechanisms in the brain. In male rats, olfactory cues are particularly important for sexual behavior. Several brain areas are key for the processing of sexually-relevant olfactory stimuli, in particular the medial amygdala (MeA), bed nucleus of the stria terminalis (BNST), and the medial preoptic area (mPOA). These areas also play crucial roles in generating copulatory behavior. Sexual experience is another important factor that improves subsequent sexual behavior and renders males more resistant to the detrimental effects of damage to the aforementioned brain areas.

In an effort to identify the brain areas in which changes occur as a result of sexual experience, immunohistochemistry was used to visualize the presence of the immediate early gene (IEG) Arc, which is indicative of activity-dependent synaptic plasticity. Sexually naïve and experienced male rats were either placed in the mating arena alone, with an inaccessible estrous female, or with a receptive female with which they could copulate on the test day. Patterns of Arc and c-Fos expression in their brains were then examined.

Sexual experience reduced latencies to mount, intromit, and ejaculate, and also increased the frequency of intromissions during copulation. As expected, copulation induced c-Fos expression in the posterior dorsal MeA, posteromedial BNST, and central mPOA regardless of prior experience. Arc expression was induced by copulation much more widely throughout the anterior BNST, posterior BNST, and MeA, as well as in the posterior mPOA, but not in the central mPOA. Surprisingly, Arc induction did not vary based on prior sexual experience, indicating that neural plasticity induced by copulation is important for both sexually naïve and experienced males. Correlations between measures of sexual behavior and IEG induction revealed that increased Arc in the BNST of naïve males was associated with higher mount latencies and numbers of mounts, while increased Arc in the MeA and mPOA of naïve males was associated with higher intromission latencies and numbers of intromissions. This suggests that Arc induction may be particularly important for improving behavior in naïve males that perform poorest.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

The study of copulation is unique because it enables researchers to simultaneously examine sex differences in, and hormonal influences on, behaviors that engage brain circuits involved in motivation and reward. Many sensory systems contribute information that is important for successful copulation; in male rats, chemosensory, or olfactory, stimuli are especially crucial (Edwards & Davis, 1997; Meisel, Lumia, & Sachs, 1980; Wang & Hull, 1980). A variety of brain areas play a role in both the processing of sex-relevant olfactory information and the generation of sexual behavior. The most crucial nodes in the male rat brain circuit for sexual behavior and sexually relevant olfactory cues are the medial amygdala (MeA), the bed nucleus of the stria terminalis (BNST), and the medial preoptic area (mPOA) (Claro et al., 1995; De Jonge et al., 1992; Heimer & Larsson, 1966). The original research presented here involves all three of these brain areas.

Additionally, many studies have found that prior sexual experience improves copulatory efficiency and protects against detrimental effects of brain damage (Bermant & Taylor, 1969; Fleming & Kucera, 1991; Lumley & Hull, 1999). Despite this, sexual behavior and relevant brain regions traditionally have not served as model systems for the study of plastic changes in neural connections. Recently, more studies have begun to investigate the neural basis of experience's performance-enhancing effects (McHenry et al., 2012; Nutsch et al., 2014). Such changes are also a focus of the present study.

A variety of techniques have been used to identify the brain areas and circuits important for male sexual behavior. In lesion studies, the brain area of interest is ablated and resulting deficits in sexual behavior provide clues as to the function of that brain area in a healthy animal (e.g., Dominguez et al., 2001). Conversely, stimulation studies use excitatory electrical stimuli to enhance activity in a specific brain area and observe any

copulatory enhancements that occur as a consequence of stimulation (e.g., Paredes et al., 1990). Microdialysis and pharmacological studies have revealed both the roles of many neurotransmitters in a variety of brain areas in copulation and which receptors mediate the effects of those neurotransmitters (e.g., Dominguez & Hull, 2010). Immunohisotochemistry studies have also been particularly helpful for phenotyping neurons in sex-relevant brain areas and for determining specifically which neural populations in these brain areas are active during sexual behavior through the examination of immediate early genes (e.g. Gréco et al., 1998).

Immediate early genes have been used extensively to study brain areas involved in male sexual behavior. The presence of either c-Fos mRNA or its protein product, for example, is indicative of changes in cellular activity resulting from exposure to a stimulus of interest. Although c-Fos is the most commonly examined IEG in studies of sexual behavior (reviewed in Pfaus & Heeb, 1997), mRNA and protein products of other genes also serve as indicators of different stimulus-induced molecular processes. One such gene is Arc, the presence of which is associated with the induction of plastic changes in the dendrites resulting from stimulus-induced activity (Bramham, et al., 2008; reviewed in Guzowski et al., 1999).

HORMONAL MODULATION OF SEXUAL BEHAVIOR.

Males and females differ consistently and dramatically in sexual behavior. Sex steroid hormones play a crucial role in both organizing and maintaining these sex differences. The pre- and peri-natal surge in testosterone, which is aromatized into estradiol (E2) in the brain, results in the masculinization and defeminization of the male rat brain via activation of estrogen receptor (ER) α and ERβ (Kudwa et al., 2006), respectively. Later, the increase in testosterone levels that occurs during puberty activates male sexual

behavior. Castration, which reduces circulating testosterone to undetectable levels relatively quickly (Krey & McGinnis, 1990), eventually impairs sexual behavior as well, but over a much longer period of time (Davidson, 1966). Testosterone treatment begins to restore sexual behaviors in castrated males relatively quickly, but extended administration is required to fully restore sexual behavior (Beach & Holz-Tucker, 1948). Testosterone itself, however, is not the crucial hormone for activation of male sexual behavior. Both E2, which is produced via aromatization of testosterone, and DHT, which is produced by reduction of testosterone and activates androgen receptors exclusively and much more strongly than testosterone itself (Wilbert, Griffin, & Wilson, 1983), are important for male sexual behavior. E2 activates most male sexual behaviors, but DHT specifically facilitates ejaculation and other appetitive aspects of sexual behavior (Vagell & McGinnis, 1997). Although the role of sex steroid hormones in sex-relevant brain areas is not specifically addressed here, several of the studies that helped identify these brain areas have also examined hormonal activation.

OLFACTORY INPUT.

Of all the stimuli that contribute to copulatory responses, olfactory and chemosensory cues are perhaps the most important for the male rat. Lesions of the olfactory bulbs severely impair copulation in naïve (Wang & Hull, 1980) and experienced male rats (Edwards & Davis, 1997; Meisel, Lumia, & Sachs, 1980). Experienced males, however, are more resistant to the effects of such lesions than are naïve males (Bermant & Taylor, 1969), suggesting that sex-relevant olfactory cues are particularly important during the rat's first copulatory experience. Additionally, deafferentation of the entire olfactory bulb does not affect experienced male rats' preference for an estrous female over a non-receptive female (Edwards et al., 1997), and deafferentation of the main olfactory bulb (MOB), but

not the vomeronasal organ (VNO), reduces the number of non-contact erections in response to estrous females (Kondo, Tomihara, & Sakuma, 1999). Both copulation and exposure to bedding from an estrous female's cage increase c-Fos expression only in the accessory olfactory bulb (AOB) (Kelliher et al., 1999) and elimination of both VNO and olfactory epithelium (OE) input to the olfactory bulb reduces c-Fos induction in the AOB and other brain areas to which it projects. Surprisingly, however, male rats with nonfunctional OE showed deficits in sexual behavior while males subjected to VNO removal did not (Dhungel et al., 2011). Finally, although olfactory stimuli are certainly important for copulation, arousing stimuli in general, such as a tail pinch or a flank shock, can induce copulation in both naïve (Wang & Hull, 1980) and experienced (Meisel et al., 1980) male rats that originally show disrupted sexual behavior resulting from olfactory bulbectomies.

MEDIAL AMYGDALA.

Lesion studies.

The olfactory bulbs project to the MeA, an area that plays a key role in processing sex-relevant olfactory cues and in copulation more generally. Lesions of the MeA in experienced males eliminate the benefits of pre-exposure to an estrous female, which normally decreases the latency of all copulatory behaviors and the number of mounts before ejaculation (De Jonge et al., 1992). Studies that examine non-contact erections, which males normally display when in the presence of a receptive female but not otherwise engaged in any consummatory sexual behaviors, and reflexive erections, which result from experimenter-administered tactile stimulation of the penis, demonstrate the importance for the MeA specifically for erections occurring in response to a sexually receptive female. Indeed, MeA lesions reduce the number of non-contact erections in experienced males, but do not affect reflexive erections (Kondo, Sachs, & Sakuma, 1997), suggesting that the MeA

is important specifically for the integration of sexually relevant olfactory stimuli. In the aforementioned study, MeA lesions also interfered with copulation more generally by decreasing the rate at which males mounted and intromitted and increasing the amount of time between intromissions. Furthermore, in sexually naïve males, MeA lesions abolish sexual behavior altogether, while cortical amygdala lesions cause less severe deficits and basolateral amygdala lesions have no effect (Kondo, 1992).

C-Fos expression and neurochemistry.

Studies using c-Fos as an indicator of neural activity also demonstrate the importance of the MeA for male sexual behavior. In general, both investigation of female odors and ejaculation, and sometimes other consummatory sexual behaviors, increase the numbers of c-Fos-immunoreactive cells in the MeA (Baum & Everitt, 1992; Baum & Wersinger, 1993; Bressler & Baum, 1996; Kelliher et al., 1999; Kippin, Caine, & Pfaus, 2003; Oboh, Paredes, & Baum, 1995; Veening & Coolen, 1998). These c-Fos increases may be a result of exposure to female odors in general rather than estrous odors specifically, as exposure to bedding from the cages of anestrous females similarly results in increased c-Fos levels in the MeA (Bressler & Baum, 1996). C-Fos expression following copulation in the MeA might be induced specifically in neurons containing androgen receptors (AR). Gréco and colleagues (1998) found that almost all MeA neurons that expressed c-Fos after mating also contained AR. Manipulation of AR activity can also influence sex-related behaviors in male rats. For example, injection of an AR antagonist into the MeA reduces non-contact erections in response to estrous females (Bialy et al., 2011). Finally, activity in different parts of the MeA may correspond to specific components of sexual behavior. For example, when ejaculation latency is artificially reduced by stimulating certain serotonin (5-HT) receptors with systemic injections of a 5-HT_{1A} receptor agonist, c-Fos

induction is specific to the lateral posterior dorsal MeA (Coolen et al., 1997), suggesting that this part of the MeA is important for ejaculation specifically, while other areas may be more important for mounts and intromissions.

BED NUCLEUS OF THE STRIA TERMINALIS.

The bed nucleus of the stria terminalis (BNST), which receives olfactory information both indirectly via projections from the MeA and directly via projections from the olfactory bulbs, is also important for copulation and processing olfactory information in the context of sexual behavior. Lesions of the posteromedial BNST increase ejaculation latency in both experienced and naïve males (Claro et al., 1995). While the MeA shows increases in c-Fos in response to female odors generally, the BNST may be specifically responsive to estrous female odors (Bressler & Baum, 1996). Regardless, investigation of a female reliably causes increases in BNST c-Fos expression (Baum & Everitt, 1992; Kippin et al., 2003; Veening & Coolen, 1998), and copulation causes at least an equivalent, and sometimes a larger, increase in c-Fos (Baum & Wersinger, 1993; Coolen et al., 1997; Kelliher et al., 1999; Robertson et al., 1991). As in the MeA, sex steroid hormones likely influence activity related to copulation in the BNST. Castrated males, for example, have higher intracellular levels of the neurotransmitter dopamine (DA) in the BNST than do intact males (Du, Lorrain, & Hull, 1998), perhaps as a result of decreased activity-dependent DA release. As was true in the MeA, systemic injection of a 5-HT_{1A} receptor agonist to trigger ejaculation after minimal sexual activity results in c-Fos expression specifically in the rostral and ventral posterior medial BNST, but not in other subregions (Coolen et al., 1997). Thus, activation of parts of the BNST is associated with ejaculation only, while other parts play a role in copulation more generally.

MEDIAL PREOPTIC AREA.

The final brain area considered here that is crucial for sexual behavior and sensitive to olfactory cues is the medial preoptic area (mPOA). The mPOA receives direct and indirect input from brain areas that process multiple types of sex-relevant sensory stimuli, including olfactory cues. Olfactory information reaches the mPOA via direct projections from both the MeA and the BNST (Simerly & Swanson, 1986). The mPOA, in turn, has reciprocal connections with these brain areas, making top-down modulation of these sensory inputs possible (Simerly & Swanson, 1988). Electrophysiological recordings from the mPOA during copulation reveal that subsets of mPOA neurons are active during all phases of sexual behavior, from pursuit and investigation though genital grooming after ejaculation (Shimura, Yamamoto, & Shimokochi, 1994). Furthermore, most neurons are active during more than one type of sexual behavior.

Lesion studies.

Lesions of the mPOA reduce male rats' preference for estrous over anestrous females (Hurtazo & Paredes, 2006). mPOA lesions also dramatically reduce, and sometimes completely eliminate, mounting, intromission, and ejaculation in experienced male rats (Brackett & Edwards, 1984; Hansen et al., 1982; Heimer & Larsson, 1966; Nie et al., 2011; Szechtman, Caggiula, & Wulkan, 1978). Inactivation of the mPOA using lidocaine infusions also reduces the percentage of males showing sexual behaviors and increases behavior latencies in males that still do (Hurtazo, Paredes, & Ågmo, 2008). In the same study, transient inactivation also reduced the amount of time males spent investigating an inaccessible estrous female, suggesting that the mPOA also plays a role in sexual motivation. Most rats do not show recovery of sexual behavior after mPOA lesions, and any improvements in those that do recover occur within the first month after the lesion (Ginton & Merari, 1977). The various subdivisions of the mPOA may be involved in

different aspects of copulation; one subregion that has received particular attention is the sexually dimorphic nucleus (SDN), which is one of few brain areas that is consistently larger in male rats than female rats. While dorsal mPOA lesions dramatically, and ventral mPOA lesions slightly, reduce copulatory behavior in experienced males, lesions in the SDN and the anterior dorsal mPOA have no effect (Arendash & Gorski, 1983). In naïve males, SDN lesions initially decrease sexual performance, but after repeated testing the sexual performance of lesioned males becomes similar to that of sham lesioned males (De Jonge et al., 1989). In the same study, however, naïve males continued to show deficits in sexual behavior under suboptimal conditions for copulation, such as presentation with a relatively less sexually receptive female. Houtsmuller and colleagues (1994) found that inhibition of aromatase activity prenatally or just after birth, which masculinizes male rat pups, decreased both the size of the SDN and the frequencies of different sexual behaviors. However, it is possible that the deficit in copulation these rats displayed could be due to the lack of pre- or perinatal estrogen in other brain regions. Interestingly, housing male rats given mPOA lesions before puberty with other males or females, as opposed to alone, lessens deficits in copulation resulting from the lesion (Klaric, 1990; Leedy et al., 1980).

Stimulation studies and c-Fos expression.

Just as mPOA lesions consistently impair sexual behavior, stimulation of the mPOA enhances it. Kindling stimulation triggers normal copulatory behavior in males that initially did not display any (Paredes et al., 1990), and just one kindling-like stimulus in the mPOA is enough to induce sexual behavior in non-copulating males (Paredes, Portillo, & Basanez, 2003). Additionally, kindling stimulation in the mPOA can induce male sexual behavior in ovariectomized, testosterone-treated female rats (Domínguez-Salazar et al., 2003). Stimulation of the mPOA does not cause sexually exhausted males to resume

copulation, however, and neither does local reduction of GABA signaling (Rodríguez-Manzo et al., 2000). Electrical stimulation of the mPOA, particularly the posterior mPOA (Giuliano et al., 2010), elicits erections (Marson & McKenna, 1994), and erections generated in this way are facilitated by pharmacological enhancement of cGMP and decreased by the reduction of NO signaling (Sato, Zhao, & Christ, 2001).

Studies examining c-Fos expression resulting from different sexual behaviors also illustrate the importance of the mPOA for copulation. Exposure to estrous female odors (Bressler & Baum, 1996; Kippin et al., 2003) increases mPOA c-Fos levels, and mating causes an even larger increase in mPOA c-Fos expression (Kelliher et al., 1999; Veening & Coolen, 1998). Experienced male rats also have higher c-Fos levels in the mPOA after copulation than naïve males, in addition to shorter mount and ejaculation latencies (Lumley & Hull, 1999). This mating-induced increase in c-Fos may not be hormone-dependent, as males castrated one week prior to a copulation test show similar c-Fos induction regardless of whether or not they received any of a variety of hormone replacement regimens (Baum & Wersinger, 1993). Although c-Fos levels in the mPOA consistently increase as a result of copulation, not all studies find increases after investigation of an estrous female or enhanced increases in experienced versus naïve males (e.g., Lopez & Ettenberg, 2002).

Neurochemistry in the mPOA.

Pharmacological and microdialysis studies have identified several neurotransmitters both inside and outside of the mPOA that influence male sexual behavior. Dopamine release in the mPOA is essential for normal copulation in male rats. Injections of DA antagonists into the mPOA interfere with most aspects of consummatory, and some aspects of appetitive, sexual behavior (Pfaus & Phillips, 1991; Warner et al., 1991). Conversely, injection of general DA agonists in the mPOA enhances sexual

performance and increases erections in male rats (Hull et al., 1986; Pehek, Thompson, & Hull, 1989; Scaletta & Hull, 1990). Local inhibition of DA uptake also facilitates erections (Adachi et al., 2003). A lower dose of the DA agonist apomorphine injected into the mPOA, which has little effect on sexual performance in control males, enhances performance in males previously given 6-OHDA injections that result in DA neuron death (Bitran et al., 1988), suggesting that removal of autoreceptor-related inhibition of release makes males more sensitive to the facilitative effects of DA in the mPOA. Within 24 hours of 6-OHDA administration, males have recovered from any deficits in copulation resulting from the lesion; even after recovery, however, deficits again become apparent if DA synthesis is inhibited (Bazzett et al., 1992). Copulation also increases levels of the DA metabolites DOPAC and HVA in the mPOA, as does exposure to an estrous female after an initial copulation (Hull et al., 1993; Mas et al., 1995)

Sex steroid hormones also influence male rat sexual behavior in part by modulating DA activity in the mPOA. Two weeks after castration, DA levels in males not receiving testosterone replacement no longer increase in mPOA during precopulatory exposure to an estrous female, which does increase DA levels in intact males and males castrated one week prior (Hull et al., 1995). Additionally, unlike males in the other experimental groups, none of the two-week castrates without testosterone replacement copulate with the female or show the increase in mPOA DA associated with copulation. Testosterone treatment, however, is not sufficient to restore copulation (Meisel, 1983) or preference for an estrous female (Edwards & Einhorn, 1986) in castrated males with mPOA lesions. In another study, all males given ten testosterone replacement injections beginning three weeks after castration resumed copulation and had increases in mPOA DA levels, while five injections was only sufficient in some rats and two injections did not restore either measure in any male (Putnam et al., 2001). The reduction in mPOA DA that follows castration may be due

to reduced release, because castrated males show increased amphetamine-stimulated DA release in the mPOA compared to intact males despite having lower basal levels, and tissue punches indicate increased intracellular DA levels in a variety of brain areas in castrates (Du et al., 1998).

Activation of D1-like DA receptors, which stimulate the excitatory secondary messenger adenylate cyclase, and D2-like DA receptors, which either inhibit or do not affect adenylate cyclase activity, in the mPOA have different effects on sexual behavior (Kebabian & Calne, 1979). A D1 agonist in the mPOA facilitates ejaculation, and antagonism of mPOA D1 receptors eliminates enhancements in sexual behavior that result from administration of the D1 agonist (Markowski et al., 1994). Indeed, administration of a D1 antagonist alone in the mPOA increases mount and intromission latencies and reduces the number of ejaculations; D2 agonist administration has the same effect (Hull et al., 1989). Both D1 and D2 antagonists decrease measures of sexual motivation, but a high-affinity D3/moderate-affinity D2 agonist enhances measures of both sexual motivation and performance (Moses et al., 1995). D1 activation also has a different effect on penile reflexes than D2 activation. While activation of D1 receptors alone facilitates reflexive erections, activating only D2 receptors specifically increases seminal emissions (Hull et al., 1992). A D2 agonist in the mPOA, both alone and in combination with a D1 antagonist, facilitates penile reflexes and, at high doses, seminal emissions (Bazzett et al., 1991). Using an agonist specific for D3 receptors, Kitrey and colleagues (2007) found that activation of that particular DA receptor subtype induced ejaculation, and that this effect was blocked by a D3 receptor antagonist.

While dopamine in the mPOA generally enhances male sexual behavior, serotonin generally inhibits it. Injection of 5-HT in the mPOA reduces the number of ejaculations and increases ejaculation latency as well as the post-ejaculatory interval (PEI) (Dominguez

& Hull, 2010). In the same study, 5-HT administration attenuated the increased glutamate release in the mPOA that normally accompanies ejaculation. In another experiment, males that had elevated levels of 5-HT in the mPOA either failed to ejaculate or, in some cases, to display any copulatory behaviors (Hull et al., 1993). Although 5-HT itself or a 5-$HT_{1B/C}$ agonist in the mPOA increases the total number of mounts needed and the time required to reach ejaculation, a 5-HT_{1A} agonist decreases the same measures (Fernandez-Guasti et al., 1992). After intracerebroventricular injection of a toxin that targets 5-HT neurons, the 5-HT_{1A} agonist 8-OH-DPAT substantially reduces mount and intromission frequency and ejaculation latency while a 5-HT_{1B} agonist increases ejaculation latency (Fernández-Guasti & Escalante, 1991). The facilitative effects of 8-OH-DPAT, however, seem at least partially due to its agonist activity at D2 receptors in addition to 5-HT_{1A} receptors (Lorrain et al., 1999). Furthermore, injection of 8-OH-DPAT into the mPOA increases local levels of both DA and 5-HT (Lorrain, Matuszewich, & Hull, 1998), which might enhance the effects of this particular agonist on sexual behavior. Finally, increased 5-HT levels in the anterior lateral hypothalamic area (aLHA), but not in the mPOA, are associated with an increase in the PEI, and injections of an SSRI into the aLHA, but not the mPOA, increases mount, intromission, and ejaculation latencies (Lorrain et al., 1997).

Pharmacological evidence also suggests that glutamate and GABA release in the mPOA affect male sexual behavior. Injection of an NMDA receptor antagonist in the mPOA interferes with sexual behavior in both naïve and experienced male rats and eliminates the enhancements in copulation that usually result from prior exposure to estrous females in naïve males (Vigdorchik et al., 2012). Conversely, injection of $GABA_A$ antagonists into the mPOA reduce ejaculation latency and PEI while $GABA_A$ agonists reduce the percentage of males displaying a variety of sexual behaviors (Fernández-Guasti, Larsson, & Beyer, 1986).

Although their effects are somewhat more subtle compared to the above neurotransmitters, injections of different opioids into the mPOA alter sexual behavior in specific ways. For example, morphine, which binds most strongly to μ-opioid receptors (MOR), increases the length of the second PEI and reduces the percentage of rats that continue to copulate after their second ejaculation, while low doses of dynorphin, which activates κ-opioid receptors, decrease latency to a second ejaculation and the intromission frequency during the second bout of copulatory behavior (Band & Hull, 1990). Morphiceptin, another MOR agonist, interferes slightly with both sexual behavior and motivation by increasing latencies to intromission and to reach a receptive estrous female in the goal box of an x-maze (Matuszewich et al., 1995). MOR activation in the mPOA may play a role in sexual reward as well; injections of the MOR antagonist naloxone block conditioned place preference for ejaculation without affecting any measures of sexual behavior (Agmo & Gómez, 1993).

Norepinephrine (NE) in the mPOA can also affect sexual behavior via activation of α and β NE receptors. Injection of NE itself enhances all measures of sexual behavior, while an α_1 blocker and, to a lesser extent, a β blocker inhibit some of the same measures (Mallick, Manchanda, & Kumar, 1996). Conversely, an α_2 agonist in the mPOA inhibits, and an antagonist facilitates, copulation, and injections of either drug in the mPOA at least partially reverse the effects of systemic administration of the opposing drug (Clark, 1991).

Manipulations that reduce nitric oxide (NO) signaling in the mPOA also interfere with sexual behavior. Injection of a NO synthase (NOS) inhibitor, for example, reduces the frequencies of mounts, intromissions, and ejaculations, and eliminates increases in these same frequencies that are normally observed during copulation when males have been pre-exposed to estrous females (Lagoda et al., 2004). On the other hand, injection of an NO precursor into the mPOA increases mount rates (Sato et al., 1998). Injection of an NO

precursor also increases DA levels in the mPOA, and this increase is blocked if a NOS inhibitor is injected simultaneously (Lorrain & Hull, 1993). Conversely, injection of a NOS inhibitor into the mPOA during copulation prevents the normal increase in DA levels that typically accompanies this behavior (Lorrain et al., 1996). Injection of a chemical that breaks down and releases NO into the mPOA of castrated males given only DHT replacement restores copulation, and the increase in mPOA DA levels that accompanies it, in some males (Sato, Wersinger, & Hull, 2007). Hormones likely modulate NO activity in the mPOA; for example, castration reduces the number of neurons in the mPOA that express a specific type of NOS, and injections of T restore expression of the same protein to levels seen in intact males for at least two months after castration (Du & Hull, 1999). Estrogen, in addition to T, regulates NOS expression, and differential regulation of NOS in the mPOA by E and T is sex-specific, allowing for a variety of hormonal effects on NO signaling both between males and females and among different brain areas (Scordalakes, Shetty, & Rissman, 2002).

SEX BEHAVIOR CIRCUIT.

Though all of the brain areas discussed thus far are important for male sexual behavior in their own regard, functional connections among these regions, and with the mPOA in particular, are also crucial for copulation. Unilateral mPOA lesions unsurprisingly reduce ejaculation-induced c-Fos in the lesioned mPOA, but also in the ipsilateral BNST (Baum & Everitt, 1992). While a unilateral mPOA lesion slightly but significantly decreases mount and intromission frequencies, the addition of a contralateral MeA lesion almost completely abolishes these behaviors (Kondo & Arai, 1995), suggesting that communication between these brain areas is essential for copulation. Increasing MeA glutamate levels with microinjections of glutamate and a glutamate uptake

inhibitor triggers DA release in the mPOA (Dominguez & Hull, 2001). Additionally, bilateral MeA lesions almost completely eliminate intromission and ejaculation, but apomorphine injections targeting the mPOA restore both behaviors to control levels in lesioned animals, demonstrating the importance of DA release in the mPOA as a consequence of MeA neural activity (Dominguez et al., 2001). The same study also found that MeA lesions abolish the increase in mPOA DA levels that usually accompanies olfactory exposure to, and copulation with, an estrous female.

PRIOR EXPERIENCE FACILITATES COPULATION.

While numerous studies show that sexual experience improves sexual behavior and confers resistance to disruption of normal neural activity related to copulation, the mechanisms by which experience alters this behavior remain largely unknown. Because of its crucial function within the male sexual behavior brain circuit, studies of neural changes resulting from sexual experience have focused on the mPOA. Fleming and Kucera (1991) found that systemic injections of either the protein synthesis inhibitor cyclohexidine or the NMDA antagonist MK-801 immediately following an initial copulation session blocks the facilitation of subsequent sexual behavior. Similarly, systemic administration of MK-801 just prior to repeated exposures to an inaccessible estrous female blocks the facilitation that usually follows such exposures during initial copulation (Powell, Dominguez, & Hull, 2003). This confirms that molecular and cellular processes associated with plastic changes in other brain regions might be responsible for changes in the mPOA as well. NMDA receptor activation is crucial for the induction of LTP (Gilbert & Mack, 1990), but NMDA antagonists also interfere with copulation itself. For example, animals given mPOA microinjections of aCSF and animals given MK-801 during an initial copulation test both show improvement in copulation when tested with re-injection of the same substance after

gaining sexual experience (Vigdorchik et al., 2012). In the same study, however, the few naïve males who copulated after MK-801 injection had fewer intromissions and ejaculations than naïve males given aCSF, and sexually experienced males given both aCSF and MK-801 in a repeated measures design showed fewer intromissions and ejaculations and had longer mount and intromission latencies after administration of the latter.

NMDA receptors are activated by glutamate release during the normal course of sexual behavior, and mechanisms controlling glutamate levels in the mPOA might also be altered by copulation to facilitate future performance. For example, in sexually naïve animals only, the number of astrocytes in the mPOA is negatively correlated with ejaculation latency (Will et al., 2015). This correlation is driven by similar correlations in the central and caudal, but not rostral, regions mPOA when it is divided into three distinct levels. Given the role of astrocytes in synthesizing new and taking up released glutamate (Hertz et al., 1999; Rothstein et al., 1996), changes in astrocyte density might affect sexual behavior by altering glutamatergic tone in the mPOA.

Expression of improved sexual behavior resulting from plastic changes accompanying experience might partially depend on DA signaling in the mPOA. Administration of a D1 antagonist in the mPOA at the time of a copulation test eliminates the enhancements in sexual behavior male rats usually display as a result of prior sexual experience (McHenry et al., 2012). Additionally, systemic administration of a D1 antagonist reduces mating-induced mPOA c-Fos in naïve, but not experienced, males (Lumley & Hull, 1999), perhaps suggesting that D1 activation contributes most to experience-induced changes during early sexual experiences.

Other studies have identified experience-dependent increases in NO release and synthesis, which in turn enhance DA release, in the mPOA (Lorrain & Hull, 1993; Lorrain

et al., 1996). Sexual experience elevates NOS levels in the mPOA, as measured by both immunohistochemistry and Western blots, regardless of whether or not males copulated on the day of sacrifice (Dominguez et al., 2006). Additionally, sexually experienced animals have more NOS-containing cells in the mPOA than naïve males regardless of whether they copulate on the day of sacrifice, while animals that copulate on the day of sacrifice have more mPOA c-Fos than those that don't regardless of prior experience (Nutsch et al., 2014). However, the number of NOS containing cells activated in naïve males that copulate on the test day is higher than in experienced males, which in turn have higher levels of colocalization than males that do not copulate prior to sacrifice. Perhaps, then, increased activity in NOS-containing neurons that follows and initial sexual experience is unique in that it initiates processes that boost NO tone in the long-term, and this lasting change enhances DA release that facilitates copulation in experienced males. Finally, injections of the NOS inhibitor L-NAME cause more disruption of sexual behavior in naïve males than they do in experienced males (Lagoda et al., 2004), indicating that sexual experience must also alter behavior through other mechanisms in addition to NO signaling.

Neural signals mediated by neurotransmitters other than DA and glutamate are also altered by sexual experience. For example, males that copulate on the day of sacrifice have higher oxytocin receptor (OTR) mRNA levels than males that don't regardless of prior sexual experience, but experienced males have higher levels of OTR protein than naïve males regardless of whether copulation occurred before sacrifice (Gil et al., 2013). In the same study, injections of OT in the mPOA reduce the first, and tend to reduce the second, ejaculation latency. It is likely that additional copulation-induced changes in signaling mechanisms for other neurotransmitters have yet to be identified.

SEXUAL BEHAVIOR AND ARC EXPRESSION.

While c-Fos is used as a general indicator of neural activation related to an experimental stimulus, including copulation (Pfaus & Heeb, 1997), expression of the immediate early gene Arc is specifically indicative of plastic changes occurring in synapses activated by stimulus presentation (Bramham et al., 2008). Recently, Arc expression has been used to provide insight into the location of experience-dependent changes in studies of sexual behavior. In female rats, paced mating induces Arc expression in the ventrolateral part of the hypothalamic ventromedial nucleus, and dendritic spine density is reduced in the same area several days later (Flanagan-Cato et al., 2006). Arc induction also occurs in the hippocampus and subdivisions of the amygdala only in response to mating paradigms that induce pseudopregnancy in female rats (Yang, Oberlander, & Erskine, 2007). Inhibition of Arc upregulation following mating in the hypothalamic arcuate nucleus of female rats resulted in similar levels of sexual receptivity in naïve versus experienced females, which otherwise showed reduced receptivity (Christensen, 2012). Arc expression has been underutilized in the study male sexual behavior, and it seems likely that Arc levels might also provide useful information about molecular processes that are initiated as a result of copulation or sexually-relevant stimuli. For example, Matsuoka and colleagues (2002) demonstrated that exposure to female pheromones induced Arc expression in the AOB and MOB, but mating caused an additional increase in the AOB only. This mating-induced Arc increase in the AOB might indicate that changes in synaptic structure specifically in this area are important in improving transmission of sex-relevant olfactory cues in experienced animals. By examining Arc expression after either olfactory investigation or copulation in brain areas that play a role in processing both of these stimuli, namely the BNST, MeA, and mPOA, the experiment presented here may help identify which areas are important for linking olfactory stimuli with copulation. Additionally,

examining differences in Arc activation between sexually experienced and naïve male rats may help identify experience-dependent differences in plasticity within this brain circuit. Finally, changes in c-Fos-positive cell counts in the BNST, MeA, and mPOA were also examined in order to both replicate previous c-Fos findings and allow for the comparison of cellular activation and induction of neural plasticity resulting from copulation.

Chapter 2: Materials and Methods

SUBJECTS

Adult male Long-Evans rats (PN 58-64, 225-249g upon arrival, N=63, Harlan Laboratories, Indianapolis, IN) were single housed in a temperature-controlled room (22ºC, 40-50% humidity) with a reverse light/dark cycle (14 hours light/10 hours dark, lights off at 10 AM). Adult female Long-Evans rats (PN 70-89, 200-224g, N=26, Harlan Laboratories) were double-housed in a separate room from males under otherwise identical conditions. Animal husbandry, surgeries, and experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin and were in accordance with the National Health Guidelines for the Use of Animals. Prior to all experiments and after approximately one week of acclimation to the animal colony, female rats were ovariectomized via ventral midline incisions under ketamine/xylazine hydrochloride (50mg/kg and 4mg/kg, respectively; Animal Health Intl., Greeley, CO) anesthesia using aseptic surgical procedures. Briefly, two ventral midline incisions were made through the skin and muscle wall, the ovaries externalized, the uterine horns ligated, the ovaries excised, the muscle wall sutured to close the incision, and the incision in the skin closed with surgical staples. Females were injected with gentamicin antibiotic (5mg/kg, RXV Products, Westlake, TX), ketoprofen (5mg/kg, Fort Dodge Animal Health, Fort Dodge, IA), and 1-2 mL of sterile 0.9% saline (Hospira, Inc., Lake Forest, IL) to assist in recovery. At least one week after ovariectomy surgeries, females were given alternating subcutaneous injections of estradiol (E, 0.2 mg/mL in sesame oil, 0.2 mL injection per animal) and progesterone (P, 0.02 mg/mL in sesame oil, 0.2 mL injection per animal) every other day to restore sexual receptivity. After approximately two weeks of hormone replacement injections, females that received P injections on the morning of the experimental procedures were used as stimulus females. Before exposure to experimental

males, female sexual receptivity was confirmed by placing them with a sexually experienced male and verifying that they exhibited full lordosis behavior in response to mounts or intromissions.

SEXUAL BEHAVIOR TESTING

All behavioral activities occurred under red light during the dark phase of the light cycle in rectangular glass testing arenas (51cm long x 26 cm wide x 32cm high) that each contained a wire mesh basket (27cm long x 14cm wide x 15cm high) suspended from the back side 17 cm above the floor and were covered by wire mesh lids. All male rats were placed alone in the arenas on four consecutive days for 30 minutes each to habituate to the testing environment. Male rats were then divided into two groups: "experienced" (n=30) and "naïve" (n=33). Over a two week period, experienced males were placed in the testing arena with a receptive female on 5 separate occasions and were allowed to copulate with her for 30 minutes on the first four occasions and for 60 minutes on the final occasion. Naïve males were treated identically, with the exception that they were placed in the testing arena alone on all occasions. During the final sexual experience session, experienced males were observed to confirm that they were able to resume intromission after ejaculation at least once within the 60 minute timeframe.

On the day of sexual behavior testing, both the experienced and naïve male groups were further divided into three additional groups (see Figure 1): "alone," "female," or "sex." Animals in the alone groups (naïve alone: n=8; experienced alone: n=8) were placed in the testing arena alone for 60 minutes. Animals in the female groups (naïve female: n=10; experienced female: n=11) were placed in the testing arena for 60 minutes along with a receptive female placed in the wire mesh basket within the testing arena. These males could see the female and access olfactory stimuli, but could not copulate with her.

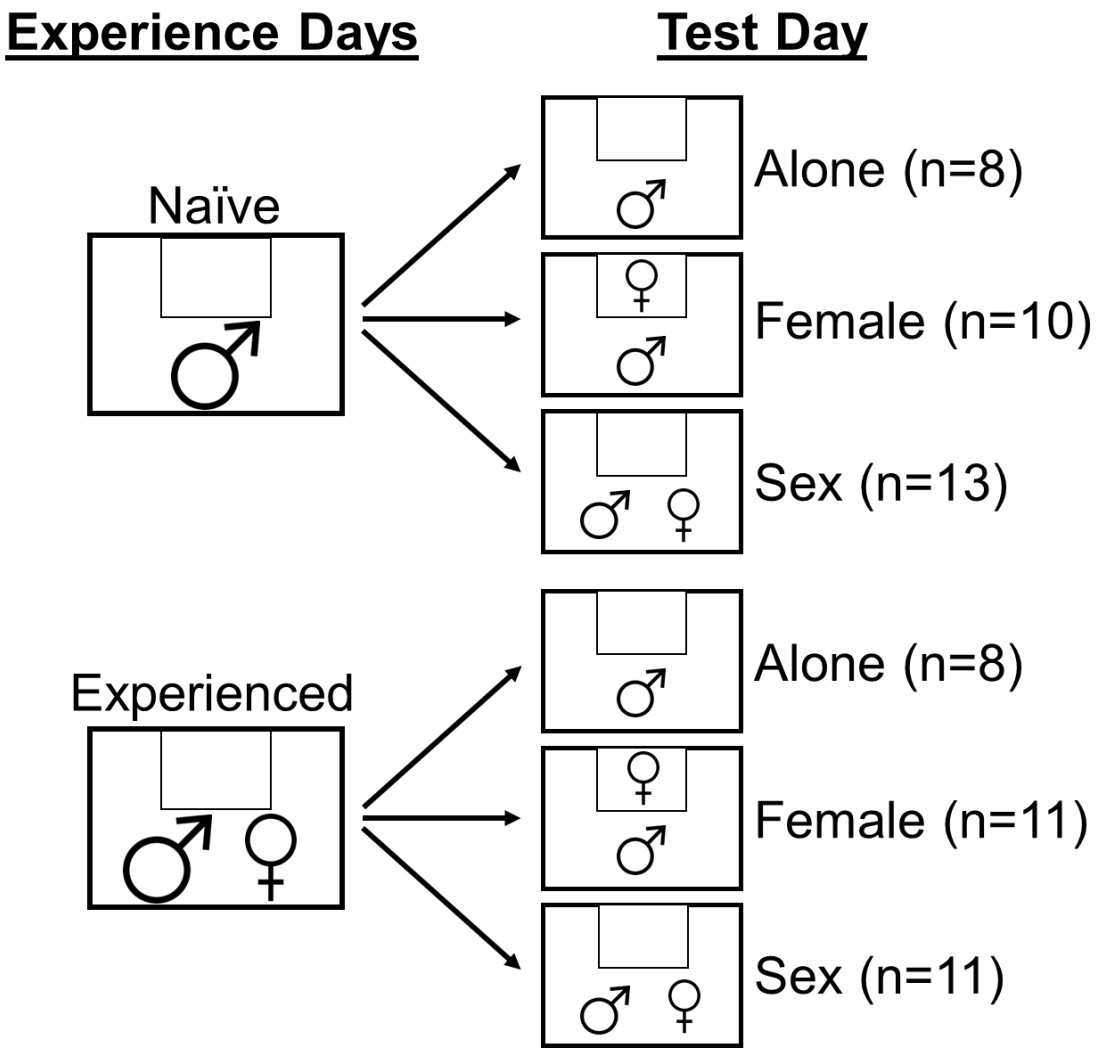

Figure 1. Experimental design. Over the course of five experience days (30 minutes for the first four days, 60 minutes on the final day), naïve males were placed in an empty testing arena and experienced males placed in the arena with a receptive female with which they could copulate. On the test day, naïve and experienced males were further divided into three experimental groups based on the stimulus to which they were exposed: "Alone" group males were placed in an empty testing arena for 60 minutes; "Female" group males were placed in the testing arena for 60 minutes with an inaccessible estrous female isolated in a wire mesh cage; and "Sex" group males were placed in the arena with a receptive female with which they could copulate and were removed upon resuming intromission after the first ejaculation.

Finally, males in the sex groups (naïve sex: n=15; experienced sex: n=11) were placed in the testing arena with a receptive female with which they could copulate and were removed after their first post-ejaculatory intromission. Two rats in the naïve sex group that did not ejaculate after 60 minutes in the testing arena with the female were removed from the experiment, leaving the naïve sex group with a final n of 13. During test sessions with sex group males, observers quantified the following measures of sexual behavior: latency to display and total number of mounts and intromissions, ejaculation latency, or the time between the first intromission and the first ejaculation, and the length of the post-ejaculatory interval, or the time between the first ejaculation and the next mount or intromission behavior. Additionally, mount and intromission frequencies were calculated for each animal by dividing the total number of behavioral displays by the time between the first intromission and the first ejaculation in minutes.

TISSUE COLLECTION

60 minutes after either removal from the testing arena (alone and female groups) or the first ejaculation (sex groups), males were injected with a lethal dose of Euthasol (0.3 mL/animal, Virbac Animal Health, Inc., Fort Worth, TX). They were then perfused transcardially with 100 mL of 0.1M phosphate buffered saline (PBS) followed by 500mL of 4% paraformaldehyde in 0.1M PB (filtered, pH 7.35). Brains were then removed, post-fixed in 4% PFA for 1 hour, transferred to 30% sucrose, and stored at 4ºC for at least 48 hours prior to sectioning. Brains were cut into 35 μm coronal sections using a freezing microtome (Microm HM 450, ThermoFisher Scientific, Waltham, MA) and sections were stored in cryoprotectant solution (30% ethylene glycol, 30% sucrose, 0.00002% sodium azide in 0.1M PB) at -20ºC until staining.

IMMUNOHISTOCHEMISTRY

Free-floating sections of brain tissue containing the BNST, pMeA, and mPOA were washed in 50mM Tris Buffer (TB) four times prior to and in between all incubations; TB also served as the diluent for incubation solutions, and all steps were done at room temperature. After the initial wash, antigen retrieval was performed by incubating sections in 10mM sodium citrate buffer (pH 8.5) at 65ºC for 15 minutes. Endogenous peroxidase activity was blocked by incubating the tissue in 1% H_2O_2 for 10 minutes. The tissue was then incubated in a blocking solution containing 0.4% Tween 20 and 5% BSA for 1 hour, after which it was transferred directly to primary rabbit anti-Arc antibody solution (156 003, Synaptic Systems, Göttingen, Germany, 1:5000 in blocking solution) and incubated overnight. The tissue was then incubated in biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, 1:1000 in blocking solution) for 1 hour. Signal amplification was achieved by incubation with avidin-biotin complex (VECTASTAIN elite ABC; Vector Laboratories, 1:1000) for one hour. Staining was visualized by incubation with 0.02% 3-3'diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO), 2% Ni2SO4 (ThermoFisher Scientific), and 30% H_2O_2 for 10 minutes. After the chromagen reaction was terminated by several TB washes, tissues were briefly transferred to 0.3% gelatin (Thermo-Fisher Scientific) solution, mounted on slides, dried, rehydrated, counterstained with 0.5% Methyl Green (Sigma-Aldrich), dehydrated, cleared in xylene, and coverslipped with DPX (VWR Intl., Radnor, PA). To verify antibody specificity, control sections were treated identically except for the omission of the primary antibody from the incubation solution, which resulted in no staining.

A second series of free-floating sections were stained for the immediate early gene c-Fos using the same procedure as described above but with the following differences: 0.1M PB was used for all washes and as diluent for all incubations that did not involve

antibodies; the antigen retrieval step was not performed; blocking solution consisted of 0.4% Triton-X and 0.5% BSA; the primary antibody used was a mouse anti-c-Fos antibody (1:5000, sc-271243, Santa Cruz Biotechnology, Inc., Dallas, TX) and the secondary antibody was biotinylated goat anti-mouse (1:1000, Vector Laboratories); Ni_2SO_4 was omitted from the chromagen solution; and tissues were not counterstained prior to dehydration and coverslip placement.

The number of Arc- and c-Fos-positive cells was quantified separately in the following brain areas, which were defined according to Paxinos and Watson (2007): lateral and medial anterior BNST (Bregma -0.24 mm), central mPOA (Bregma -0.24 mm), lateral and medial posterior BNST (Bregma -0.96mm), posterior mPOA (Bregma -0.96 mm), and dorsal and ventral posterior MeA (Bregma -2.76 mm). The area counted for each brain area is depicted in Figure 2. Each brain area was quantified on both the left and the right side for each animal; the averages of these two numbers were used for all analyses.

STATISTICAL ANALYSES

All data were analyzed using R statistical computing software (version 3.1.0, "Spring Dance"). Measurements of sexual behavior from sexually experienced and sexually naïve animals were compared using Welch's t tests. Differences in the number of Arc- and c-Fos-positive cells depending on prior sexual experience and test day stimulus were identified using two-way ANOVAs independently for each brain area. When appropriate, Tukey HSD post-hoc tests were used to evaluate differences driving significant effects. Additionally, Pearson correlation coefficients between Arc- and c-Fos-positive cell counts in all of the brain areas and the various behavioral measures of sexual performance were calculated.

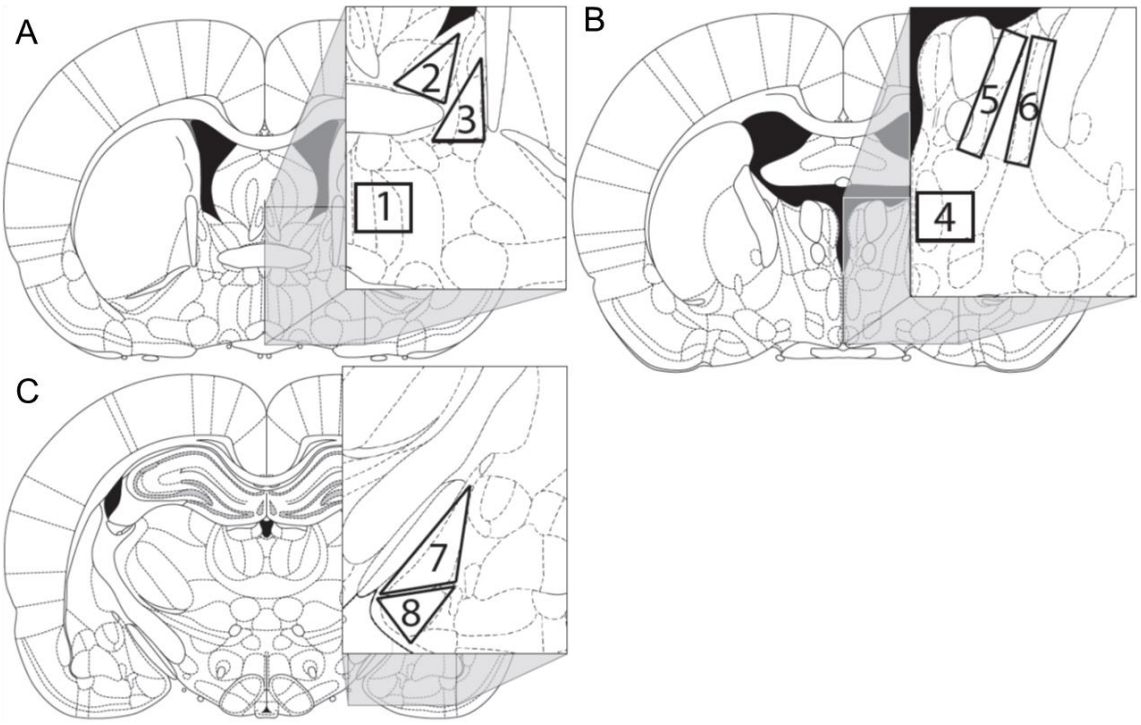

Figure 2. Brain areas examined. All brain atlas figures adapted from Paxinos and Watson (2007). (A) Figure 35 showing areas in which cell counts were quantified for 1) central mPOA, 2) amBNST, and 3) alBNST. (B) Figure 41 showing 4) posterior mPOA, 5) pmBNST, and 6) plBNST. (C) Figure 57, showing 7) pdMeA, and 8) pvMeA.

Chapter 3: Results

COPULATION INDUCES C-FOS EXPRESSION

Copulation induced c-Fos expression only in the expected brain areas, and olfactory investigation of a female induced c-Fos expression in the central mPOA only (Figure 3). In the central mPOA, there was a main effect of stimulus ($F_{(2,53)}$=22.9, p=1.05x10^{-7}) such that sex groups had more c-Fos-positive cells compared to female groups (diff=26.9, p=0.00038), which in turn had more c-Fos-positive cells than alone groups (diff=18.8, p=0.032); sex groups also had significantly more c-Fos-positive cells than alone groups (diff=45.7, p=1x10^{-7}). In the posterior dorsal MeA, a significant main effect of stimulus ($F_{(2,58)}$=9.49, p=0.00030) resulted from elevated c-Fos-positive cell counts in sex groups as compared to both female groups (diff=14.9, p=0.0027) and alone groups (diff=17.5, p=0.00089). There was no stimulus main effect in the posterior ventral MeA. Finally, in the posterior medial BNST, there was a significant main effect of stimulus ($F_{(2,56)}$=3.79, p=0.029) due to increased c-Fos-positive cell counts in sex groups compared to alone groups (diff=20.9, p=0.024). No significant main effects of stimulus were found in the anterior lateral, anterior medial, or posterior lateral BNST. In addition, no significant main effects of prior sexual experience were detected in any of the brain areas examined. No significant stimulus by experience interactions were found either, although in the anterior lateral BNST there was a trend towards such an interaction ($F_{(2,59)}$=3.03, p=0.057) due to a non-significant tendency for increased c-Fos-positive cells counts in naïve males that had sex compared to naïve males placed in the testing arena alone (diff=29.6, p=0.077).

COPULATION INDUCES ARC EXPRESSION

In contrast to c-Fos expression, significant main effects of test stimulus on the number of Arc-positive cells present were found in all but one of the brain areas examined

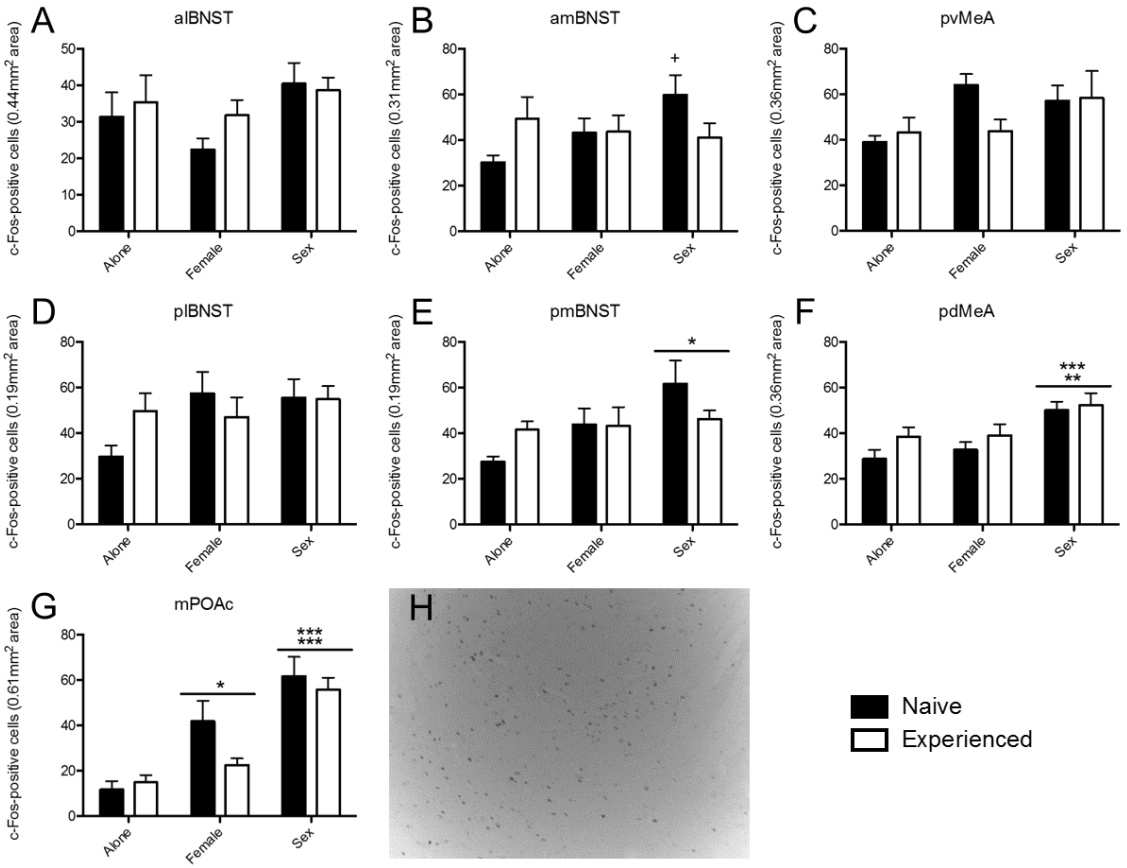

Figure 3. Copulation and exposure to female increase c-Fos-positive cell counts regardless of sexual experience. C-Fos-positive cell counts were higher in the sex groups as compared to the alone groups in the pmBNST (E). In the pdMeA (F) and mPOAc (G), c-Fos counts in the sex groups were higher than those in both the alone and female groups. In the mPOAc, c-Fos counts were also higher in female groups compared to alone groups. Finally, in the amBNST (B), there was a trend for a stimulus by experience interaction such that naïve males that had sex tended to have higher c-Fos levels than naïve alone males. There were no signficant differences, depending on either test day stimulus or experience, in c-Fos counts in the alBNST (A), pvMeA (C), or plBNST (D). A representative micrograph of c-Fos immunohisotchemical staining from the plBNST of an experienced alone group male is shown in (H). All values are expressed as mean ± SEM. $^{+}0.10>p>0.05$, $*p<0.05$, $**p<0.01$, $***p<0.001$.

(Figure 4). In both the alBNST and amBNST, the significant stimulus main effect ($F_{(2,53)}$=3.38, p=0.042 and $F_{(2,58)}$=5.39, p=0.0074, respectively) was driven by greater Arc-positive cell counts in males that had sex as compared to those exposed to females (diff=11.8, p=0.045 and diff=12.6, p=0.0083, respectively). In both posterior BNST subdivisions, the stimulus main effect (plBNST: $F_{(2,47)}$=6.48, p=0.0035; pmBNST: $F_{(2,57)}$=5.52, p=0.0067) resulted from elevated Arc-positive cell counts in sex groups as compared to both the female groups (plBNST: diff=8.06, p=0.0049; pmBNST: diff=12.2, p=0.014) and the groups placed in the testing arena alone (plBNST: diff=7.45, p=0.020; pmBNST: diff=11.3, p=0.029). Because the pattern of differences in Arc counts was identical for both the lateral and medial BNST subdivisions, these were combined to give one average Arc-positive cell count each for the anterior and posterior BNST. In the anterior BNST, a stimulus main effect ($F_{(2,56)}$=4.32, p=0.019) resulted from increased Arc expression in sex groups compared to female groups (diff=11.4, p=0.029). A significant main effect of stimulus was also found in the posterior BNST ($F_{(2,56)}$=6.85, p=0.0023), but there sex groups had more Arc-positive cells than both female (diff=10.4, p=0.0059) and alone (diff=10.0, p=0.013) groups. The stimulus main effect in the posterior dorsal MeA ($F_{(2,56)}$=10.3, p=0.00017) reflected greater Arc-positive cell counts in sex groups as compared to both female (diff=19.8, p=0.0030) and alone (diff=25.9, p=0.00044) groups. In the posterior ventral MeA, however, the stimulus main effect ($F_{(2,57)}$=6.24, p=0.0037) was driven by increased Arc-positive cell counts in sex groups as compared only to alone groups (diff=9.83, p=0.0028). Interestingly, there was a significant main effect of stimulus in the posterior mPOA ($F_{(2,60)}$=5.20, p=0.0085) but not in the central mPOA. Sex groups had increased Arc counts as compared to both female (diff=9.91, p=0.022) and alone (diff=10.5, p=0.024) groups in the posterior mPOA. No significant main effects of prior

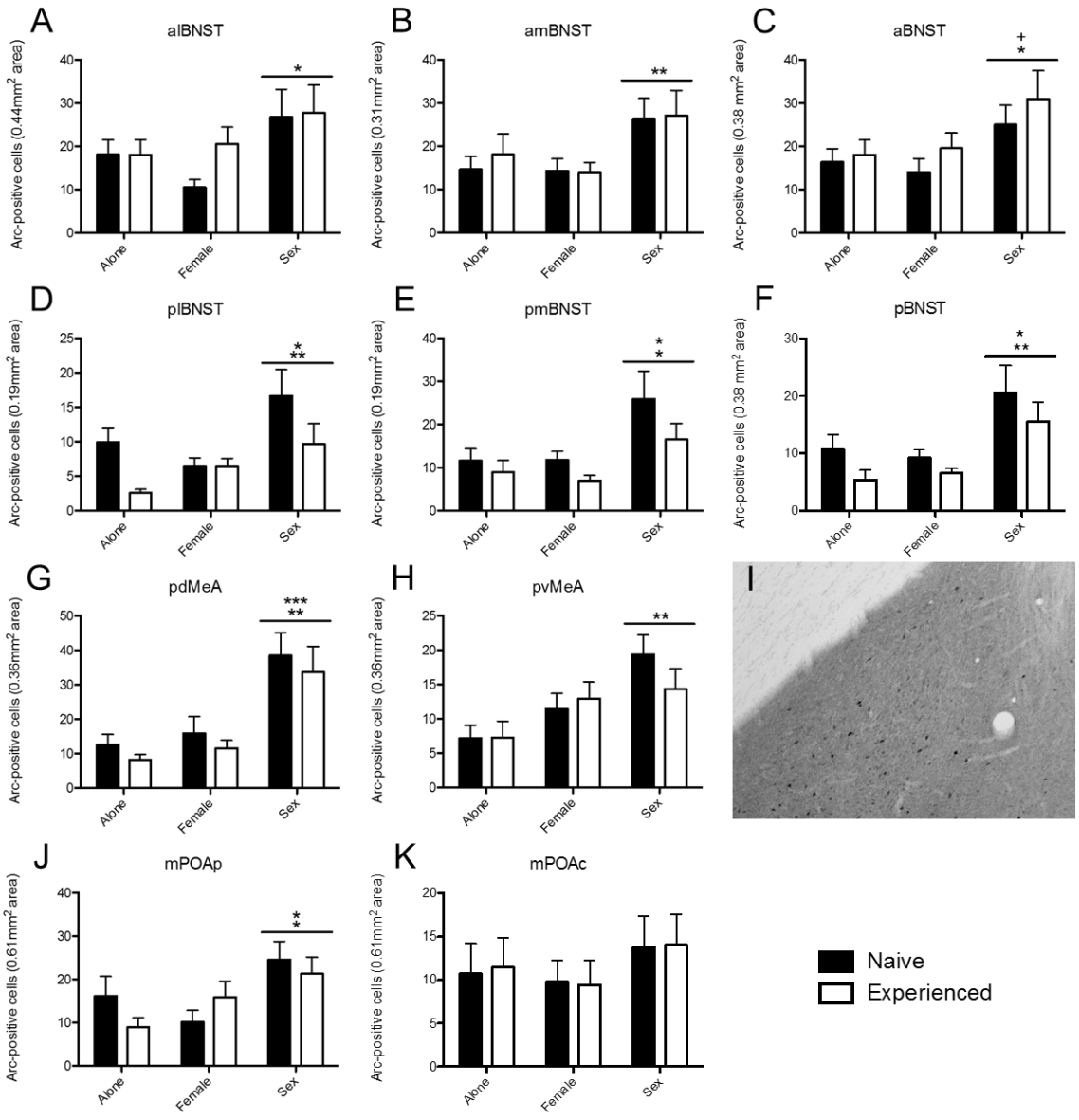

Figure 4. Copulation increases Arc-positive cell counts regardless of sexual experience. In the alBNST (A), amBNST (B), and aBNST overall (C), sex groups had more Arc-positive cells than female groups only. In the plBNST (D), pmBNST (E), pBNST overall (F), pdMeA (G), and mPOAp (J), sex groups had higher Arc counts than both female and alone groups. In the pvMeA (H), sex groups had higher Arc counts than alone groups only. Finally, in the mPOAc (K), there were no significant differences in Arc counts among the groups. A representative micrograph of Arc immunohistochemical staining from the pdMeA of an experienced sex group male is shown in (I). All values are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

sexual experience or experience by stimulus interactions were found in any of the brain areas examined.

EXPERIENCE IMPROVES SEXUAL PERFORMANCE

Sexually experienced males displayed shorter latencies to mount, intromit, and ejaculate than did sexually naïve males (mount latency: $t=3.81$, $df=10.6$, $p=0.0031$; intromission latency: $t=2.77$, $df=11.3$, $p=0.018$; ejaculation latency: $t=2.87$, $df=15.4$, $p=0.0011$). In addition, experienced males had higher intromission frequencies than naïve males ($t=2.64$, $df=19.73$, $p=0.016$; see Figure 5). There were no differences between experienced and naïve animals in the number of mounts or intromissions displayed, mount frequency, or the duration of the post-ejaculatory interval.

CORRELATIONS BETWEEN C-FOS EXPRESSION AND MEASURES OF SEXUAL BEHAVIOR

Correlations were calculated in order to determine in which brain areas individual differences in the number of c-Fos-positive cells were consistently related to differences in sexual performance. All correlations were calculated first for all copulating males combined regardless of prior experience to identify overall patterns, and then separately for naïve and experienced males to determine which group of males drove the overall effect. Significant correlations between c-Fos-positive cell counts for each brain area and sexual behavior are listed in Table 1.

CORRELATIONS BETWEEN ARC EXPRESSION AND MEASURES OF SEXUAL BEHAVIOR

The number of Arc-positive cells in both naïve and experienced males, both together and separately, were also analyzed for correlations with measures sexual behavior. Due to identical patterns in the induction of Arc expression by copulation, the lateral and medial subdivisions were again combined for both the anterior and the posterior BNST, and correlations using these combined Arc counts were also calculated. Significant

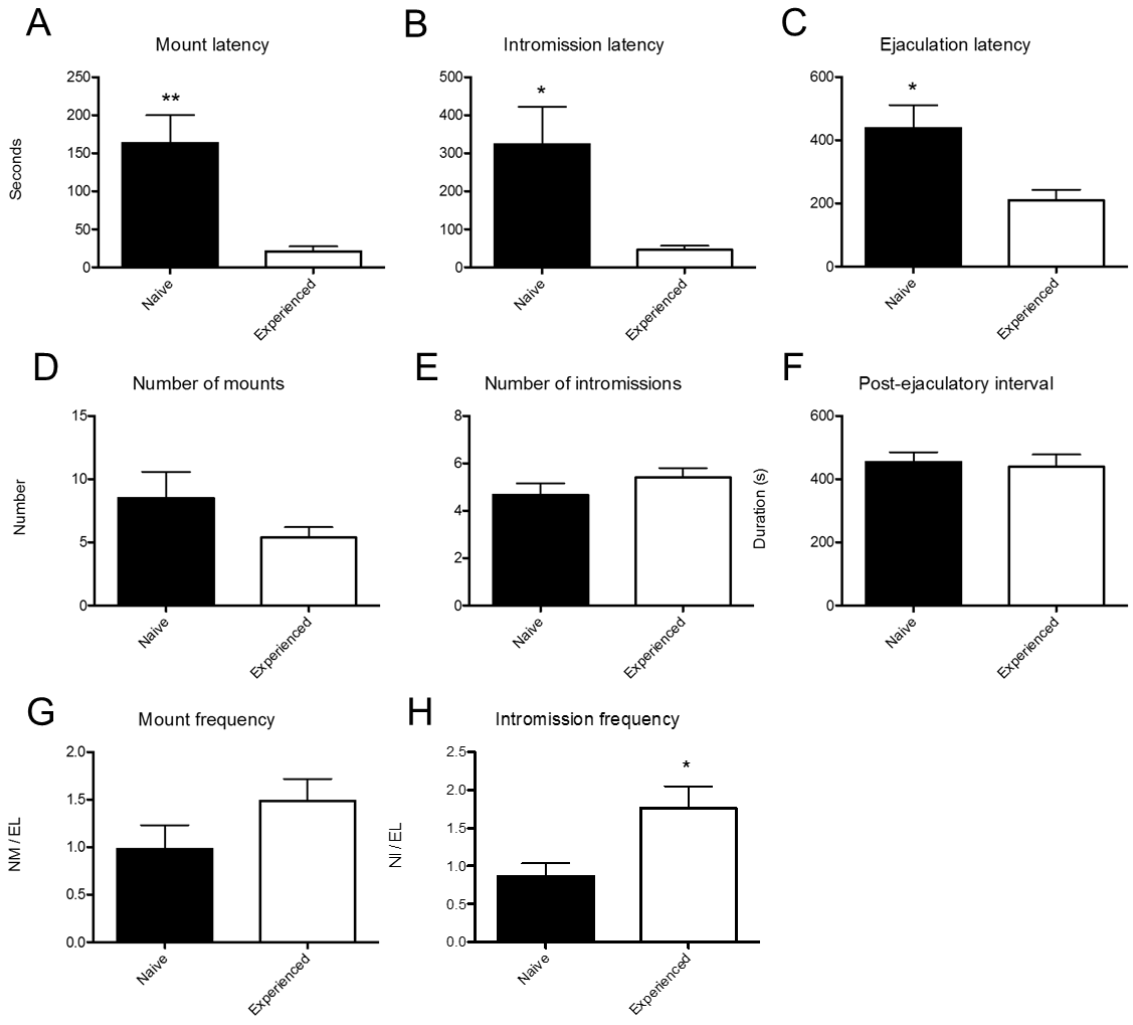

Figure 5. Sexual experience improves copulatory performance. Naïve males have higher mount (A), intromission (B), and ejaculation (C) latencies than experienced males. Experienced males, however, have higher intromission frequencies (H) during copulation than naïve males. Naïve and experienced males did not differ in number of mounts (D), number of intromissions (E), duration of the post-ejaculatory interval (F), or mount frequency (G). All values are expressed as mean ± SEM. *$p<0.05$, **$p<0.01$.

Brain area	Behavior	Experience	R	p value
alBNST	EL	Naïve	0.61	0.0339
pmBNST	ML	Experienced	-0.73	0.0400
pvMeA	PEI	Combined	0.43	0.0426
pvMeA	PEI	Experienced	0.74	0.0146
pvMeA	EL	Experienced	0.66	0.0369
pvMeA	IL	Experienced	0.82	0.0021
pdMeA	IL	Experienced	0.80	0.0028
mPOAc	MF	Experienced	0.67	0.0328

Table 1. Correlation between c-Fos and sexual behaviors. Correlations between cell counts and behaviors when all copulating males are combined regardless of experience and when males are separarted by experience are shown separately. Pearson's r and associated p-values are reported.

correlations between Arc-positive cell counts for each brain area and sexual behavior are listed in Table 2.

Brain area	Behavior	Experience	R	p value
plBNST	NM	Combined	0.73	0.0018
plBNST	NM	Naïve	0.74	0.0146
plBNST	ML	Naïve	0.68	0.0444
pmBNST	NM	Combined	0.81	<0.0001*
pmBNST	NM	Naïve	0.92	<0.0001*
pBNST	NM	Combined	0.78	<0.0001*
pBNST	NM	Naïve	0.90	<0.0001*
pBNST	ML	Experienced	0.74	0.0236
alBNST	ML	Experienced	0.68	0.0456
pvMeA	IL	Combined	0.51	0.0134
pvMeA	IL	Naïve	0.64	0.0260
pdMeA	IF	Naïve	-0.73	0.0049
mPOAc	NI	Combined	-0.54	0.0159
mPOAc	NI	Naïve	-0.72	0.0187
mPOAp	IL	Combined	0.53	0.0087
mPOAp	IL	Naïve	0.66	0.0196

Table 2. Correlation between Arc and sexual behaviors. Correlations between cell counts and behaviors when all copulating males are combined regardless of experience and when males are separarted by experience are shown separately. Pearson's r and associated p-values are reported. * indicates correlations that remain significant when α level is adjusted in an attempt to control for multiple comparisons (10 brain areas and 8 behaviors, $\alpha=0.05/80 = 0.000625$).

Chapter 4: Discussion

In this study, I examined Arc expression to identify any differences in copulation- or estrous female-induced neural plasticity between sexually experienced and naïve male rats. I also quantified c-Fos expression, which has been used extensively to study brain areas that are either activated by or drive sexual behavior (e.g., Baum & Everitt, 1992; Baum & Wersinger, 1993; Kelliher et al., 1999), in the same males. My aim in comparing c-Fos expression to Arc expression was to distinguish brain areas activated by sexual behavior from those undergoing plastic changes in response to sexual behavior.

QUANTIFICATION OF IEG INDUCTION BY COPULATION OR ESTROUS FEMALE EXPOSURE

c-Fos

The c-Fos findings reported here generally agree with prior studies in that copulation increased numbers of c-Fos-positive cells in the pmBNST, pdMeA and mPOA. In addition, investigation of an estrous female increased c-Fos-positive cell counts in the mPOA as compared to males placed in the arena alone, and this increase was smaller than the copulation-induced increase. There were no significant differences in c-Fos induction as a function of prior sexual experience. Although copulatory experience certainly both improves, and reduces the impact of treatments interfering with, sexual behavior, c-Fos induction may not be the best indicator of underlying experience-dependent changes in the brain. For example, while Lumley and Hull (1999) found greater sex-induced mPOA c-Fos levels in experienced males than in naïve males, Nutsch and colleagues (2014) found no differences based on experience. The reason for this discrepancy is unclear. One possibility is that experience-dependent changes in c-Fos expression depend crucially on the number of opportunities the male has had to copulate. Although experienced males in this experiment had six opportunities to copulate prior to sacrifice (as compared to seven

total opportunities in both Lumley and Hull and Nutsch et al.), it is possible that animals with as few as one ejaculation each on two separate occasions were included in the experienced groups.

Somewhat surprisingly, olfactory investigation of an inaccessible estrous female did not result in increased c-Fos levels in either the pdMeA or the pmBNST. However, at least one study also found that investigation of an estrous female, as opposed to an anestrous female or an empty arena, does not induce c-Fos in the BNST or mPOA in either naïve or experienced males (Lopez & Ettenberg, 2002).

Arc

More surprisingly, there was no significant effect of prior experience on copulation-induced Arc expression in any of the brain areas examined. I predicted that naïve animals would show elevated Arc expression in sex-relevant brain areas compared to experienced animals, as an up-regulation of plastic processes could help explain improvements in performance that result from an initial sexual experience. Instead, the results suggest that continuous modification of neural circuitry underlying sexual behavior might be advantageous for both naïve and experienced males. The few studies of plastic changes in sex-relevant brain areas in male rats have focused mostly on the size of neurons in specific sexually dimorphic brain regions or on the size of the brain region itself. For example, activation of both androgen and estrogen receptors contributes to increased soma size and overall volume of the pdMeA in male rats as compared to females (Cooke, Breedlove, & Jordan, 2003). Sexual behavior can also trigger large-scale morphological changes in the male rat brain. While repeated copulation with a receptive female maintains the larger size of the SDN-POA normally found in male rats as compared to females, both males repeatedly exposed to a non-receptive female and castrates have reduced SDN-POA

volumes (Prince et al., 1998). Additionally, the volume of the anteroventral periventricular nucleus (AVPV) in males placed with non-receptive females did not differ from the AVPV volume of female rats, while all other males, including castrates, had significantly lower AVPV volumes. This suggests that sexual behavior, in addition to hormones, plays a role in maintaining behaviorally significant sexual dimorphisms in the male brain.

Hormones and copulation also modulate synaptic structure in female rats. Cyclical increases in estrogen during the estrous cycle transiently alter the structure of the female arcuate nucleus to more closely resemble the male phenotype (Hung et al., 2003). The only study that has examined Arc as an indicator of sex-induced plastic changes did so in the context of female sexual behavior. Flanagan-Cato and colleagues (2006) found that paced mating increased Arc levels in the ventrolateral hypothalamic ventromedial nucleus (vlVMH) in both sexually naïve and sexually experienced females one hour after copulation. In the same study, a separate group of females had reduced spine densities on one of the three dendritic compartments examined in the vlVMH five days after mating compared to females that never copulated. This reduction in spine density does not necessarily indicate what role Arc protein plays in the affected neurons, however. Arc might help stabilize any expansion that occurred in the remaining spines as a result of LTP, but it could also be involved in endocytosis of AMPARs at spines that were eventually eliminated (Chowdhury et al., 2006; Guzowski et al., 2000). Additionally, plasticity-inducing stimuli result in increases of Arc protein levels only in the portion of the dendrite containing the activated synapses (Moga et al., 2004). Arc mRNA levels also increase in the dendrites, specifically at activated synapses, in response to plasticity-inducing stimulation (Lyford et al., 1995; Steward et al., 1998), adding to the complexity of Arc's synaptic effects. It is possible, then, that Arc might simultaneously play different roles at different dendritic locations in the same brain area in response to sexual stimuli. It might

also contribute to LTP and LTD processes differently in sexually experienced versus sexually naïve animals. Future studies examining the consequences of Arc expression within specific dendritic compartments of both naïve and experienced males will help illustrate how plastic changes in sex-relevant brain areas contribute to experience-dependent improvement in sexual behavior.

Copulation increased Arc-positive cell counts in all BNST and pMeA subregions examined as well as in the posterior mPOA. The only brain region in which Arc expression did not increase after mating was the central mPOA. This is surprising given the importance of cellular activity in both central and posterior regions of the mPOA for consummatory sexual behaviors in particular (Balthazart & Ball, 2007). Perhaps the posterior mPOA is relatively more important for copulation-induced neural plasticity than the central mPOA. Considering, for example, the different roles in appetitive and consummatory sexual behaviors played by different parts of the mPOA (Balthazart et al., 1998; Taziaux et al., 2006), and the differences in neural projections to the mesolimbic reward system in the anterior versus the posterior mPOA (Tobiansky et al., 2013), it seems reasonable to assume that plastic changes following sexual experience might differ depending on exact location within the mPOA. In the anterior BNST, Arc was elevated similarly in both the lateral and medial subregions in copulating males as compared to males placed with inaccessible females. The results in the medial and lateral posterior BNST are similar, but copulating males additionally had higher Arc expression than males placed in the arena alone. These results suggest that there is no medial-lateral gradient in copulation-induced plasticity as indicated by Arc expression. Arc levels were higher in both the pdMeA and pvMeA following copulation compared to placement in the empty arena. In the pdMeA, but not the pvMeA, Arc levels were also higher in copulating males than in males exposed to inaccessible females. Throughout the BNST and pMeA, then, Arc expression in response

to copulation was more widely distributed than the c-Fos expression observed here and in other studies. Specifically, sex-induced c-Fos expression is consistently observed only in the pmBNST and pdMeA, and is occasionally seen in the amBNST as well (Coolen et al., 1997; Coolen, Peters, & Veening, 1997; Coolen, Peters, & Veening, 1996).

Exposure to an inaccessible estrous female did not induce Arc expression in any of the brain areas examined. Given the enhancement in sexual performance such exposure typically confers (McHenry et al., 2012; Powell, Dominguez, & Hull, 2003; Vigdorchik et al., 2012), I expected to find more Arc in at least some sub-regions in response to an inaccessible estrous female. It is possible, however, that Arc induction requires both cellular activity due to ascending input generated by sex-relevant stimuli and descending input from integrative brain areas resulting from copulation itself. This seems to be the case in the AOB, where Arc expression is much higher after copulation in an estrous odor-rich environment compared to exposure to the estrous odor exposure without copulation (Matsuoka et al., 2002). This may also represent an important difference between Arc induction and c-Fos induction, which is increased to the same degree in the AOB after either exposure to estrous odors or copulation (Kelliher et al., 1999).

PRIOR EXPERIENCE IMPROVES SEXUAL BEHAVIOR

Sexual behavior latencies can be considered appetitive behavioral measures that are related to sexual motivation, while total numbers and frequencies of sexual behaviors are almost always considered consummatory sexual behaviors (Dewsbury, 1969; Everitt, 1990). In this study, sexual experience seems to have improved copulation by increasing some aspects of both sexual motivation and performance. Decreased mount and intromission latencies in experienced males indicated that they initiated copulation more quickly, while decreased ejaculation latency and more frequent intromissions in the same

males suggest an increase in copulatory efficiency. Meanwhile, numbers of mounts and intromissions and mount frequency did not differ depending on experience. This pattern of results matches almost exactly the findings of Dewsbury (1969) in one of the initial studies of sexual experience's effects on copulation, and other groups have found very similar behavioral changes (e.g. Fleming & Kucera, 1991). Some studies additionally find increases in the number of mounts and/or intromissions in experienced males (e.g. Vigdorchik et al., 2012), but these changes were not observed here. The most important experience-induced changes in behavior, however, may be the increased sexual arousal demonstrated by decreased mount and intromission latencies and the increase in copulatory performance resulting from lower ejaculation latencies and more quickly paced intromissions, both of which were observed here. Finally, perhaps due to decreased latencies to engage in copulation, experienced males are often able to achieve more ejaculations in timed copulation tests compared to naïve males (e.g. McHenry et al., 2012). However, because males in this experiment were only allowed one ejaculation before removal from the mating arena, this behavioral measure could not be examined.

CORRELATIONS BETWEEN ARC EXPRESSION AND SEXUAL BEHAVIOR

Mounting behaviors and the BNST

Generally, males that had higher Arc levels throughout the pBNST also displayed higher numbers of mounts regardless of experience. Separating males by experience revealed that the relationship between Arc in the pBNST and mount number was driven by significant positive correlations in naïve, and not experienced, males. Although some of these correlations were the strongest found, interpretation of their implications is complicated by the similar number of mounts displayed by experienced and naïve males. Perhaps Arc induction in the pBNST, which is strongest in males that require many mounts

during their first copulatory opportunity, serves to improve copulatory performance by reducing the number of mounts required to reach ejaculation during subsequent bouts of sexual behavior. This relationship might exist only in naïve animals because they vary more in number of mounts displayed, and only some naïve males reach a sufficient number of mounts to induce Arc expression that triggers improvement.

There were also more isolated positive correlations between mount latency and Arc in some BNST regions. Specifically, naïve males that had more Arc-positive cells in the plBNST also had higher mount latencies, as did experienced males with more Arc-positive cells in the alBNST and the pBNST as a whole. Naïve males had significantly higher mount latencies than experienced males, so Arc induction in the plBNST that increases as mount latencies increase in naïve males specifically might play an important role in altering neural connections to reduce mount latency and improve copulatory performance. More diffuse Arc induction throughout the BNST may additionally aid in continuous improvements in mount latency in experienced males, even though they are much better at, and less variable in, this behavior.

Intromission behaviors and the pMeA and mPOA

In the pvMeA, the positive correlation between Arc-positive cell counts and intromission latency in all males was driven by naïve, and not experienced, males. As was the case with mount latency, naïve males were both more variable in, and displayed significantly higher, intromission latencies. Perhaps, then, increased Arc induction helps reduce intromission latency in naïve males with the highest initial latencies. Additionally, when naïve males were examined in isolation, a negative correlation between the number Arc-positive cells and intromission frequency was found in the pdMeA. Naïve males intromitted at a significantly lower frequency than experienced males, and this difference

can be considered an example of improved copulatory efficiency. Arc induction, then, might signal the initiation of plastic changes that help increase intromission frequency in poorly performing naïve males. Finally, regardless of experience, males with more Arc-positive cells in the mPOAc and mPOAp tended to intromit less overall and have longer intromission latencies, respectively. Both of these correlations were driven by naïve, and not experienced, males. Arc induction in the mPOA, then, might also spur plastic changes that improve subsequent intromission behavior, although naïve and experienced males did not differ in number of intromissions during copulation.

CORRELATIONS BETWEEN C-FOS EXPRESSION AND SEXUAL BEHAVIOR

Correlations between c-Fos-positive cell counts and measures of sexual behavior were much more sporadic. Naïve males with more c-Fos-positive cells in the alBNST had higher ejaculation latencies, while experienced males with more c-Fos-positive cells in the pmBNST had lower mount latencies. C-Fos induction in the pmBNST is associated with copulation generally and ejaculation specifically (Coolen et al., 1997; Coolen et al., 1997; Veening & Coolen, 1998), so the correlation observed in this area in experienced males was not surprising. However, no relationship between copulation-induced c-Fos and alBNST activity was observed, so the association in naïve males between alBNST activity and poorer copulatory performance was unexpected. The only experience-independent significant relationship was the positive correlation between c-Fos-positive cell counts in the pvMeA and duration of the post-ejaculatory interval. This correlation, which was driven by experienced males, is also difficult to interpret, because there was no effect of sexual experience on post-ejaculatory interval and pvMeA activity was not associated with sexual behavior. Isolated positive correlations between intromission latency and c-Fos cell counts in both the pvMeA and pdMeA were found only for experienced males. Increased

activity in the pdMeA is typically associated with ejaculation (Coolen et al., 1997; Coolen et al., 1997; Veening & Coolen, 1998) and would be expected to improve sexual performance, so the direction of the latter correlation is surprising. Finally, in the mPOAc of experienced males only, higher c-Fos counts tended to accompany higher mount frequencies. Although this behavioral measure did not differ as a function of experience, it is plausible that increased mPOAc activity might contribute to relatively higher copulatory efficiency of some experienced males.

FUTURE DIRECTIONS

Several future studies could be conducted to help determine the consequences of Arc activation patterns observed here. Double labeling for c-Fos and Arc using immunohistochemistry would show to what extent cell populations activated by sexual behavior overlap with those that experience plastic changes in response to sexual behavior. For example, more overlap of cellular activation and plastic changes in naïve animals might help strengthen neural connections resulting in successful copulation, while less overlap in experienced males might indicate fine-tuning of connections via elimination of input from inactive or weakly activated neurons. Determining the neurochemical phenotype of the neurons that express Arc after copulation would also be particularly informative. This, in combination with studies of the specific molecular pathways in which copulation-induced Arc is involved, would give tremendous insight into precisely how the sexual behavior circuit including the BNST, MeA, and mPOA is altered by sexual experience.

CONCLUSIONS

Prior experience improved sexual behavior by decreasing latencies to begin copulation and by increasing the pace of copulation such that experienced males ejaculate sooner than naïve males. This suggests that experience both increases sexual arousal and

improves specific aspects of sexual performance. The number of mounts and intromissions and the pace of mounts during mating did not change as a function of experience.

The immediate early gene Arc, which is indicative of synaptic plasticity in response to an experimental stimulus, was induced throughout the BNST, MeA, and posterior mPOA of male rats in response to copulation. Increases in Arc were more wide-spread in these brain areas than copulation-induced c-Fos levels, which were similar to those found in other studies of neural activation after copulation. Interestingly, there was no effect of prior sexual experience on copulation-induced Arc expression, suggesting that plastic changes occur throughout sex-relevant brain regions in both naïve and experienced males. Finally, olfactory cues from an estrous female, which increased c-Fos expression in the central mPOA, did not induce Arc expression in any of the brain areas examined, perhaps indicating that co-occurrence of olfactory cues and successful copulation is required for plastic changes to occur.

Correlations between IEG levels and measures of sexual behavior were found for a variety of behaviors and brain regions. Mounting and intromission behaviors were correlated with Arc-positive cell counts throughout the BNST, MeA, and mPOA; analyzing males separately based on prior experience revealed that correlations specifically in naïve animals, and not experienced animals, drive the overall correlations. Additionally, Arc levels tended to be highest in the naïve males that were worst at sexual behavior, suggesting that Arc-dependent plasticity may serve a corrective role in these animals. Finally, fewer correlations were found between c-Fos levels mostly in the MeA and primarily behavioral latencies. These correlations were driven by experienced, and not naïve, males, and c-Fos levels tended to be higher in animals with poorer sexual performance. The implications of these correlations between behavioral measures and c-Fos levels are unclear. Future studies identifying the type of plastic changes signified by the presence of Arc in response to sexual

activity would help in the development of experiments examining causal relationships between prior sexual experience, Arc and c-Fos expression, and measures of copulatory behavior.

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